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Reconstitution of the Beef Heart and Rat Liver Mitochondrial K^+/H^+ (Na^+/H^+) Antiporter

QUANTITATION OF K^+ TRANSPORT WITH THE NOVEL FLUORESCENT PROBE, PBFI*

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New indicators for fluorescent measurement of Na^+ and K^+ ions should prove particularly useful for studies of reconstituted carriers of these ions. We show that PBFI, a K^+-specific probe, provides a convenient and sensitive assay for the study of K^+ uptake mediated by the reconstituted mitochondrial K^+/H^+ (Na^+/H^+) antiporter. Fluorescent measurements have enabled us for the first time to establish reconstitution of the K^+/H^+ (Na^+/H^+) antiporter from beef heart as well as from rat liver mitochondria. This technique has also enabled us to establish that dicyclohexylcarbodiimide is capable of complete inhibition of K^+/H^+ antiport in the reconstituted system, in accord with findings in intact mitochondria. PBFI fluorescence, which measures net K^+ uptake, was essential for this corroboration, since dicyclohexylcarbodiimide is not capable of complete inhibition of $^{42}K^+/K^+$ or $^{86}Rb^+/Rb^+$ exchange, presumably because it acts selectively on proton transport within the carrier.

Mitochondrial volume homeostasis is maintained by the regulated functioning of a nonselective K^+/H^+ (Na^+/H^+) antiporter which transports all alkali cations (1–4). This carrier is inhibited reversibly by quinine and other amphiphilic amines (1–5) and irreversibly by DCCD[1] (3, 4). Reconstitution of the non-selective K^+/H^+ (Na^+/H^+) antiporter from rat liver mitochondria was recently established using $^{86}Rb^+$ uptake assays (6). This assay has several disadvantages, the most important of which is that it cannot distinguish between Rb^+/Rb^+ and Rb^+/H^+ translocation modes. Thus, Rb^+/Rb^+ exchange is only partially inhibited by DCCD, an effect that has been demonstrated in both intact mitochondria and in proteoliposomes (6, 7). On the other hand, *net* cation transport by the antiporter (cation/proton exchange) is completely inhibited by DCCD, an effect that has only been demonstrated in intact mitochondria (3, 4). For this reason, it has not yet been established whether DCCD is capable of inhibiting net cation transport via the reconstituted antiporter.

We have now explored the use of a novel potassium fluorescent indicator, PBFI. This technique has allowed us to obtain evidence for reconstitutive activity of the K^+/H^+ (Na^+/H^+) antiporter from beef heart mitochondria as well as from rat liver mitochondria, using the physiological substrate, K^+. We can now demonstrate complete inhibition of K^+/H^+ antiport by DCCD in the reconstituted system, since this technique senses net K^+ uptake. Finally, we demonstrate that propranolol and timolol inhibit K^+/H^+ exchange in the reconstituted heart preparations with I_{50} values similar to those observed in intact mitochondria (5).

EXPERIMENTAL PROCEDURES

Detergent Solubilization of Membrane Proteins—SMPs from beef heart, prepared according to Ref. 8, and from rat liver, prepared by sonication (9), were isolated by centrifugation. They were resuspended at 50 mg of protein/ml in 30 mM TEA/SO_4, and 1 mM TEA/EDTA, 10 mM TEA/HEPES, pH 7.2 (*Buffer A*), and the stock suspensions were frozen and stored at −70 °C. Thawed stock was diluted to 9.5 mg/ml with Buffer A containing Triton X-100 (3.3 mg/ml protein) and beef heart cardiolipin (3 mg/ml protein). The suspension was stirred for 20 min at 0 °C. Extracted proteins were then separated from unsolubilized material by centrifugation at 130,000 × *g* for 35 min.

Preparation of Liposomes and Reconstitution of Membrane Extracts—Liposomes were prepared essentially as described by Kakar *et al.* (6). 116 mg of asolectin (crude soybean phosphatidylcholine type IV, Sigma) was dried and dispersed in 1.65 ml of 30 mM TEA/SO_4, 1 mM TEA/EDTA, 120 mM TEA/HEPES, pH 7.4 (*Buffer B*). The fluorescent K^+ indicator, PBFI (Molecular Probes, Inc.), was added to a final concentration of 65–75 μM. The lipid suspension was then sonicated for about 3 min in a batch sonicator.

1.5 ml of the sonicated liposome suspension was mixed with 1.5 ml of a crude protein extract or with an identical solution lacking proteins. 200-μl aliquots of this mixture were placed on the tops of 1-ml Bio-Beads SM-2 (Bio-Rad) columns which had been pre-equilibrated with a 1:1 mixture of Buffers A and B. The columns were allowed to stand at room temperature for 60 min and then centrifuged at 400 × *g* using a Sorval GLC-1 tabletop centrifuge. The proteoliposomes were washed free of external probe by passage through 1-ml Sephadex G-25-300 (Sigma) columns that had been pre-equilibrated with a 1:1 mixture of Buffers A and B. The "internal medium" thus contains 30 mM TEA/SO_4, 1 mM TEA/EDTA, 65 mM TEA/HEPES, pH 7.4, and 65–75 μM PBFI. The final proteoliposome suspension usually contained 55 mg of lipid/ml and 630 μg of extracted protein/ml.

Internal volume of proteoliposomes was estimated from the distribution of [^{14}C]TEA^+ in parallel samples to which [^{14}C]TEABr was added before Bio-Beads treatment. Duplicate 50-μl aliquots were counted prior to Bio-Beads treatment and after washing out the external radioisotope by Sephadex (see above). The derived internal volume usually amounted to 0.2 μl/mg of lipid in proteoliposomes and 1.1 μl/mg of lipid in liposomes. The amount of protein actually reconstituted into the vesicles was evaluated by the Amido Black method (10).

Fluorescence Measurements—Fluorescence of PBFI-loaded proteo-

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[1] The abbreviations used are: DCCD, *N,N'*-dicyclohexylcarbodiimide; SMPs, submitochondrial particles; TEA, tetraethylammonium cation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

liposomes and liposomes was measured with an SLM 8000 fluorometer (SLM, Urbana, IL) connected to an IBM PS/2 model 60 computer. Excitation was set up at 343 nm (16 nm slit width) and emission at 500 nm (8 nm slit width). Data acquisition was performed at 0.2-s increments using an integration time of 0.1 s. 100-μl aliquots of proteoliposome suspension were transferred to a cuvette containing 1.9 ml of 150 mM KCl, 25 mM TEA/HEPES, pH 7.9 ("external medium"), and fluorescence was measured immediately.

RESULTS

The Effect of K^+ Concentration on the Emission Spectra of PBFI—PBFI emission spectra were measured while changing $[K^+]$ in a medium identical with that used in the liposome interior. Increasing $[K^+]$ caused a remarkable increase in fluorescence intensity and a continuous shift of emission maximum (Fig. 1). The wavelength of maximum emission decreased from 548 nm, at $[K^+] = 0$, to 492 nm, at $[K^+] \geq 80$ mM. Variations in [PBFI] from 10^{-6} M to 10^{-4} M caused a shift in excitation maximum from 340 nm to 360 nm. We overcame the influence of this shift by using the maximum slit width (16 nm) in excitation.

We carried out extensive screening of amphiphilic amines that inhibit the K^+/H^+ (Na^+/H^+) antiporter (2, 5) in order to identify those that caused the least interference with the PBFI assay. Fluorescence was minimally affected by timolol, butacaine, dibucaine, and propranolol, but all these amines interfered slightly with the absorbance of the probe, thereby decreasing the fluorescence intensity somewhat. Other amines, including quinine, interfered strongly with probe measurements, due to their own fluorescence and/or to direct interactions with the probe.

PBFI Fluorescence Changes in Proteoliposomes Reconstituted from Beef Heart Membranes—PBFI is an impermeant polyanion. It was found to remain inside proteoliposomes for many hours, as indicated by constant fluorescence intensity levels. As shown in Fig. 2, a rapid increase of PBFI fluorescence was observed when proteoliposomes containing internal medium plus 0.14 mM K^+ were diluted into external medium (142.5 mM K^+). This fluorescence increase was strongly inhibited by timolol and by DCCD pretreatment. Noteworthy

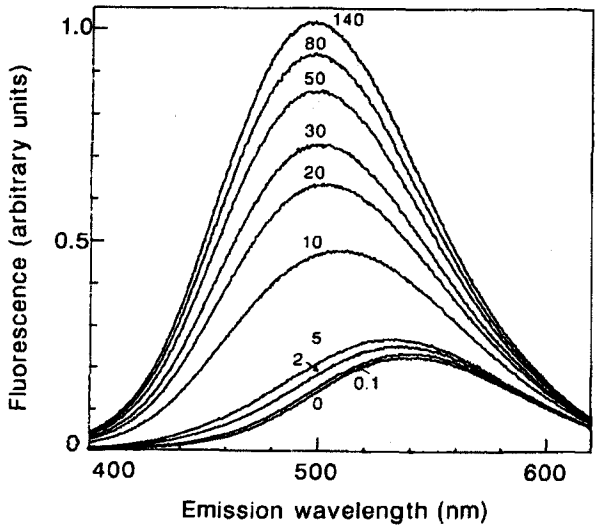

FIG. 1. **Emission spectra of PBFI at various $[K^+]$.** Fluorescence spectra were measured in internal medium (see "Experimental Procedures") to which was added KCl in concentrations (mM) indicated in the figure. For [KCl] less than 10 mM, KCl was added as aliquots of 2 M stock solution. For [KCl] greater than 10 mM, it was added as preweighed powder. Excitation wavelength was set at 360 nm, slit widths at 2 nm.

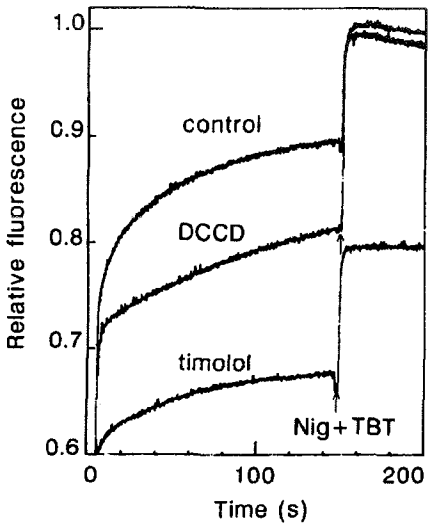

FIG. 2. **Spontaneous changes in PBFI fluorescence in proteoliposomes reconstituted from extracts of beef heart mitochondria SMPs.** The figure contains traces from three runs of a typical experiment on proteoliposomes reconstituted from extracts of beef heart mitochondria and assayed in external medium, described under "Experimental Procedures." Nigericin (0.5 μM) and tributyltin (5 μM) were added at 150 s (*Nig + TBT*). The representative conditions shown in the figure are as follows. *control*, spontaneous fluorescence changes with no additions. Proteoliposomes were pretreated with ethanol as in the DCCD assay; *DCCD*, proteoliposomes were pretreated with 2 mM DCCD in ethanol for 60 min at 25 °C; *timolol*, fluorescence changes in the presence of 0.5 mM timolol.

is the finding that DCCD-treated proteoliposomes responded rapidly to K^+ uptake after addition of a nigericin/tributyltin mixture, and the fluorescence level reached by the equilibrated preparation was nearly identical with that in control proteoliposomes. Timolol also appears to inhibit, but the compression and shift of the signal by timolol obscures its effect on raw fluorescence changes.

Calibration of PBFI Fluorescence Responses to K^+ in Liposomes—We calibrated the fluorescence response to internal $[K^+]$ in the following way: liposomes or proteoliposomes were diluted into medium containing 100 mM TEA/SO_4 and 25 mM TEA/HEPES, pH 7.9. Nigericin (0.5 μM) and tributyltin (5 μM) were added, usually causing some fluorescence decrease. Since nigericin and tributyltin catalyze K^+/H^+ and Cl^-/OH^- antiport, respectively, equilibration of KCl across the membrane is assured, and it can be assumed that $[K^+]_{in} = [K^+]_{out}$. Additions of KCl aliquots resulted in stepwise increases of fluorescence. Typical titration curves in the presence and absence of 0.5 mM timolol are contained in Fig. 3*A*.

Fluorescence intensities, $F[K^+]$, were found to depend hyperbolically on $[K^+]$. This behavior is most simply understood if $F[K^+]$ is assumed to be proportional to the concentration of K^+-probe complex, P_{K+}. That is,

$$F[K^+] = F[0] + \alpha P_{K^+} \quad (1)$$

where $F[0]$ is base-line intensity under conditions of the experiment, and α is a proportionality coefficient. The equilibrium between P_{K^+} and $[K^+]$ can be written:

$$P_{K+} = P_T/(1 + K_{eq}/[K^+]) \quad (2)$$

where P_T is total probe concentration. Equations 1 and 2 can be combined to yield the calibration equation:

$$\{F[K^+] - F[0]\} = \{F_{max} - F[0]\} - \frac{k_{eq}\{F[K^+] - F[0]\}}{[K^+]} \quad (3)$$

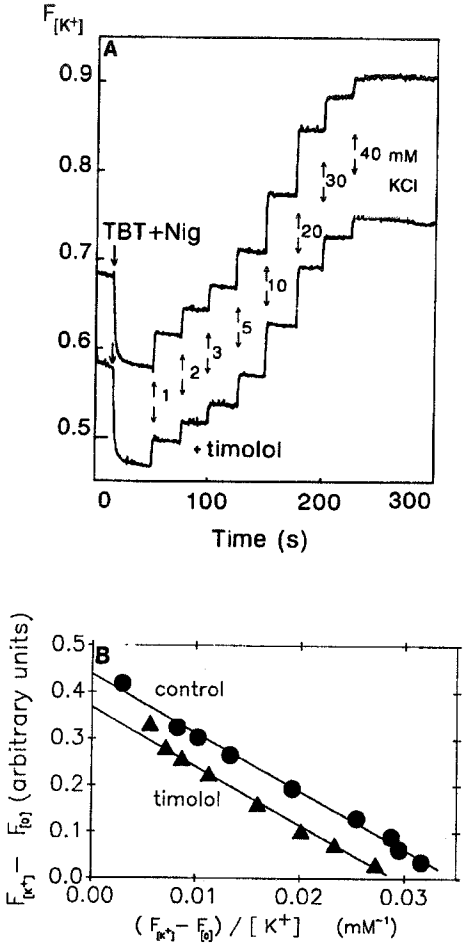

FIG. 3. **Calibration of fluorescence response of PBFI to K^+ in proteoliposomes.** Proteoliposomes were reconstituted from extracts of beef heart mitochondria SMPs, as described under "Experimental Procedures." Proteoliposomes (5.47 mg of lipid) were added to a cuvette containing 1.9 ml of 100 mM TEA/SO_4, 25 mM TEA/HEPES, pH 7.4. After 15 s, a mixture of nigericin and tributyltin chloride was added (*TBT + Nig*) at final concentrations of 0.5 μM and 5 μM, respectively. At times indicated by *arrows*, aliquots of 2 M stock KCl solution were added to the indicated final concentrations. Fluorescence was measured with excitation at 343 nm (16 nm slit width) and emission at 500 nm (8 nm slit width). Data acquisition was performed at 0.2-s increments with 0.1 s integration time. *A*, fluorescence titration records in the presence and absence of 0.5 mM timolol. *B*, linearization of PBFI fluorescence response to K^+. Data from *A* were plotted according to Equation 3. The slopes of the lines, by linear regression, yielded k_{eq} values of 12.5 mM in *control* and 12.7 mM in the samples containing 0.5 mM *timolol*.

where F_{max} is fluorescence intensity at $k_{eq}/[K^+] \rightarrow 0$.

We calibrated the K^+ response in four preparations of proteoliposomes. Fig. 3*B* contains data from Fig. 3*A* plotted according to Equation 3. The lines in Fig. 3*B* are parallel, indicating that timolol does not affect binding of K^+ to PBFI. We also observed that DCCD does not affect k_{eq} (not shown). The derived k_{eq} is 12.5 mM, which compares well with a value of 10 mM in pure solution obtained by Minta and co-workers (data sheet from Molecular Probes, Inc.).

Equation 3 was inverted and used to solve for $[K^+]$ (t) during carrier-mediated transport in proteoliposomes. k_{eq} and the span, $F_{max} - F[0]$, were obtained from calibration runs carried out on the same preparation. F_{max} was estimated from the measured $F[K^+]$ after addition of nigericin plus tributyltin at the end of the run in 142.5 mM K^+. A small, initial transient increase in apparent $[K^+]_i$ was observed in all traces. Control experiments established that this was due to mixing of *external* probe, which was not completely removed from the vesicles, with high medium $[K^+]$.

K^+ Uptake into Liposomes—It was important to establish that K^+ uptake into liposomes is sufficiently low and that it responds appropriately to ionophores. Fig. 4 contains representative experiments on two liposome preparations. Results are expressed in $[K^+]_{in}$, using Equation 3. Background rates of K^+ uptake are very low, confirming that the lipid bilayer is poorly permeable to K^+. Addition of the K^+/H^+ ionophore, nigericin, results in a rapid K^+ uptake (Fig. 4, *curve b*). Addition of the K^+ uniport ionophore, valinomycin, causes a slight stimulation of rate, not readily apparent in the short interval portrayed in the figure. This uptake is presumably due to $\Delta\Psi$-driven H^+ leak from the vesicles, and its low rate reflects the low conductance of the lipid membrane to protons. The rate of proton leak can be quantitated with longer sampling times (not shown). Addition of the protonophore, CCCP, after valinomycin, thereby providing a counterion transport pathway, causes rapid K^+ uptake. Addition of CCCP alone (not shown) results in rates of K^+ uptake much lower than those observed with valinomycin alone, testifying to the low K^+ permeability of this preparation. From these results, we conclude that PBFI is suitable for measuring K^+ uptake into vesicles. Furthermore, when liposomes are prepared with the same protocols as proteoliposomes, their membranes are shown to present good barriers to H^+ and K^+ uniport.

K^+ Uptake into Proteoliposomes Reconstituted from Beef Heart and Rat Liver Submitochondrial Particles—Fig. 5*A* contains data from PBFI fluorescence when PBFI was trapped in proteoliposomes reconstituted from rat liver membranes. A rapid spontaneous K^+ uptake was observed that was not stimulated by CCCP (Table I) and was inhibited by DCCD. This experiment confirms reconstitution of K^+/H^+ antiport from liver mitochondria (6), but it extends the earlier study in three respects. (i) The physiological substrate, K^+, is assayed instead of Rb^+. (ii) Uptake is much faster, reflecting the higher $[K^+]_o$ that can be used with this technique. (iii) DCCD inhibits nearly 100%, reflecting the fact that this technique senses *net* K^+ uptake on the antiporter, which is completely inhibited by DCCD in intact mitochondria (3, 4).

Fig. 5*B* contains data from PBFI fluorescence when PBFI was trapped in proteoliposomes reconstituted from beef heart membranes. This is our first reconstitution of the heart K^+/H^+ antiporter, which also exhibits inhibition by timolol and DCCD. DCCD is capable of complete inhibition of K^+ uptake

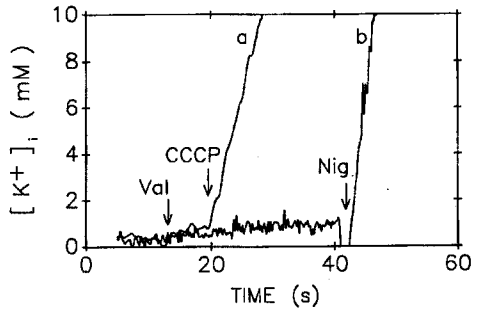

FIG. 4. **Ionophore-induced K^+ uptake in liposomes.** Data from fluorescence of PBFI trapped in protein-free liposomes are expressed as $[K^+]_{in}$, calculated as described under "Results." Liposomes were suspended in external medium, pH 7.2. *Trace a*, 1 μM valinomycin (*Val*) and 90 nM CCCP (*CCCP*) were added at times indicated by *arrows*. *Trace b*, 0.5 μM nigericin (*Nig*) was added at the time indicated by *arrow*.

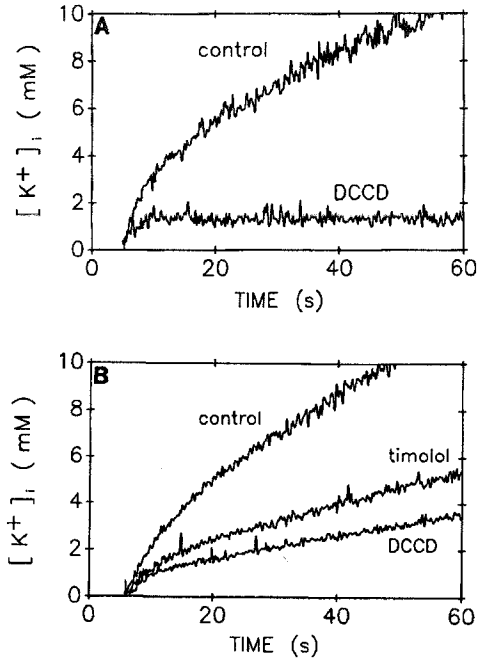

FIG. 5. **Spontaneous and inhibited K^+ uptake in proteoliposomes reconstituted from extracts of beef heart and rat liver mitochondria.** Data of the type shown in Fig. 2 were transformed into traces expressed as intraliposomal [K^+] according to calibrations described under "Results." Assay media were identical with that described in the legend to Fig. 2. Initial rates of K^+ uptake into proteoliposomes are contained in Table I. *A*, proteoliposomes reconstituted from extracts of rat liver mitochondria. *Control*, no additions, but proteoliposomes were preincubated for 70 min at 25 °C with ethanol vehicle. *DCCD*, proteoliposomes were pretreated with 2 mM DCCD in ethanol for 120 min at 25 °C. *B*, proteoliposomes reconstituted from extracts of beef heart mitochondria. *Control*, no additions, but proteoliposomes were preincubated for 60 min at 25 °C with ethanol vehicle. *timolol*, 0.5 mM timolol was added to assay medium. *DCCD*, proteoliposomes were pretreated with 2 mM DCCD in ethanol for 60 min at 25 °C. In all these traces, nigericin + tributyltin caused rapid K^+ uptake, as in Fig. 2 (not shown).

TABLE I

Comparison of K^+ uptake in proteoliposomes reconstituted from beef heart and rat liver mitochondria

Condition	Initial K^+ uptake rate	*n*	% control
	nmol K^+/(min mg protein)		
Beef heart			
Control	497 ± 20	11	100
90 nM CCCP	540	1	108
2 mM DCCD, 60 min	115 ± 15	5	23
0.5 mM timolol	210 ± 15	3	42
Rat liver			
Control	387 ± 14	2	100
90 nM CCCP	384	1	99
2 mM DCCD, 60 min	52 ± 27	2	14

after prolonged exposure (90–120 min) of proteoliposomes to this agent (not shown). K^+ uptake rates for beef heart and rat liver K^+/H^+ antiporter are compared in Table I. The rates are somewhat faster in heart than in liver, but the results are otherwise similar.

CCCP caused a negligible stimulation of K^+ uptake (Table I), confirming that this K^+ transport is electroneutral. Addition of 100 nM valinomycin after CCCP caused nearly instantaneous equilibration of K^+ (not shown), showing that the dose of CCCP used was sufficient to equilibrate $\Delta\tilde{\mu}_{H^+}$.

The difference between carrier-mediated K^+ uptake and K^+ leak becomes more apparent when compared as fluxes per unit area, since liposomes are considerably larger. On this basis, K^+ uptake into liposomes was less than 3% of that into proteoliposomes.

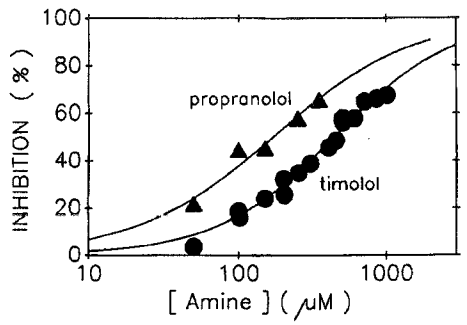

FIG. 6. **Dose-response curves for inhibition of K^+ uptake in proteoliposomes by the amphiphilic amines propranolol and timolol.** Initial rates of K^+ uptake in mM/min obtained from initial slopes of data traces such as those of Fig. 5 were obtained in the presence of various concentrations of *timolol* and *propranolol*. Rates are expressed as percent of duplicate controls without inhibitor. Hill plots of these data (not shown) yielded slopes of 1.07 and 0.93 for timolol and propranolol, respectively. The respective IC_{50} values were 400 μM and 170 μM, and the *curves* were plotted using these values.

K^+ uptake by the reconstituted beef heart K^+/H^+ antiporter was inhibited by propranolol as well as timolol (Fig. 5*B*). Fig. 6 contains dose-response curves for these two inhibitors. The I_{50} values for timolol (443 μM) and propranolol (170 μM) compare reasonably well with the values of 810 μM and 70 μM, respectively, obtained with intact rat liver mitochondria (5).

DISCUSSION

This is the first application of the new fluorescent indicator for potassium, PBFI, to K^+ transport across biomembranes. Our results show that PBFI is suitable for rapid, efficient, and sensitive assay of K^+ transport in liposomes. When trapped in proteoliposomes reconstituted with inner membrane proteins, the probe responds rapidly and appropriately to K^+ uptake mediated both by reconstituted antiporter and by nigericin (Fig. 2). Calibration curves based on total fluorescence are reproducible and well behaved, and k_{eq} for K^+ is in a suitable range (Fig. 3). Data from liposomes (Fig. 4) demonstrate very low K^+ flux despite a 10^3-fold excess of $[K^+]_o$ over $[K^+]_i$. Results with CCCP and valinomycin suggest that PBFI may prove useful for studying H^+/OH^- leak (11) in liposomes in the presence of K^+ and valinomycin.

We used PBFI to adduce evidence for reconstitution of the K^+/H^+ (Na^+/H^+) antiporter from beef heart as well as from rat liver mitochondria (Figs. 2, 5, and 6). Presumptive evidence for successful reconstitution is provided by the finding that DCCD and timolol inhibit K^+ uptake, properties expected from studies in intact mitochondria (1–5, 7). We observed that prolonged exposure to DCCD was capable of inhibiting K^+ uptake to the low levels seen in liposomes. Furthermore, the I_{50} for timolol inhibition is similar to that observed in intact mitochondria (5). These results strongly indicate that the observed K^+ uptake into the reconstituted proteoliposomes is mediated by the nonselective K^+/H^+ (Na^+/H^+) antiporter.

Conclusive evidence for involvement of a carrier is obtained from the finding that K^+ uptake is not stimulated by the protonophore, CCCP (Table I). There are several possibilities for K^+ uniport pathways in this preparation, including endogenous K^+ leak pathways (11), K^+ uniporter pathways (12, 13), or Triton-mediated increases in K^+ conductance (6, 14). Any

such leak pathways, however, would become manifest when H^+ conductance is made very high by addition of protonophore. We conclude that K^+ uniport is negligible in this preparation and that the observed K^+ uptake is obligatorily electroneutral, *i.e.* mediated by the electroneutral K^+/H^+ (Na^+/H^+) antiporter (15).

The demonstration that DCCD is capable of complete inhibition of the reconstituted K^+/H^+ (Na^+/H^+) antiporter from both beef heart and rat liver is of central importance to characterization of this carrier. Through protein labeling by [^{14}C]DCCD under highly selective conditions, we identified an 82-kDa protein as being responsible for K^+/H^+ (Na^+/H^+) antiport in mitochondria (3), and we are now pursuing the purification and characterization of this protein (16). On the basis of the finding that DCCD does not fully inhibit $^{42}K^+/K^+$ exchange in mitochondria, Brierley and co-workers (17) questioned whether this agent blocks the K^+/H^+ antiporter. We have demonstrated that DCCD is a *partial* inhibitor of $^{86}Rb^+/Rb^+$ exchange, both in intact liver mitochondria (6, 7) and in the reconstituted system (6). We have also shown that DCCD is capable of *complete* inhibition of net K^+ uptake into mitochondria (3, 4). We pointed out (4) that these findings are consistent with a mode of action in which DCCD inhibits proton transport but not cation/cation exchange through the antiporter. The new evidence that DCCD completely inhibits net electroneutral K^+ uptake into proteoliposomes (Fig. 5) provides strong support for this contention.

Cation-specific fluorescent probes offer several advantages over isotope exchange assays in establishing reconstitution of cation porters in mitochondria. They provide a continuous record, rather than discrete time points; they are more sensitive, allowing more assays to be carried out on a given proteoliposome preparation; and the assays are less time-consuming. For the study of the K^+/H^+ (Na^+/H^+) antiporter, fluorescent probes offer particular advantages that derive from the properties of the carrier itself. (i) We can assay in the physiological range of [K^+], whereas $^{86}Rb^+/Rb^+$ exchange is best assayed at 1 mM Rb^+, far below the apparent K_m for Rb^+ of 100 mM (7). (ii) Physiological substrates of the carrier, namely K^+ and Na^+, can be studied. Indeed, we have observed DCCD inhibition of Na^+ uptake, using the Na^+-specific probe, SBFI.[2] (iii) The probe only detects *net* K^+ uptake, enabling us to demonstrate complete DCCD inhibition.

All of these advantages result in a more sensitive and more straightforward assay of K^+/H^+ antiport in reconstituted proteoliposomes. The fluorescence technique will now be used to characterize the properties of the purified protein[3] and to test the hypothesis that this protein mediates mitochondrial K^+/H^+ and Na^+/H^+ antiport (3).

[2] S. Nath, P. Ježek, and K. D. Garlid, unpublished data.

[3] X. Li, M. Hegazy, F. Mahdi, P. Ježek, R. D. Lane, and K. D. Garlid, manuscript in preparation.

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